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IκBL, a novel member of the nuclear IκB family, inhibits inflammatory cytokine expression

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ABSTRACT

We previously reported that IκBL prevents experimental autoimmune arthritis. The molecular mechanism, however, still remains unclear. In contrast to four splicing-isoforms of IκBL in human, two isoforms were identified in mouse. The major isoform IκBL-α(S) suppressed LPS-induced NF-κB activation and transcription of TNFα and IL-6, but not IL-1β. The suppressive activity required the nuclear localization signal and the ankyrin repeat domain of IκBL. IκBL did not affect the nuclear translocation of the NF-κB dimer. These findings point to IκBL as being a novel member of the nuclear IκB family, which functions in the nucleus and controls various inflammatory responses including autoimmune arthritis.

1. Introduction

Nuclear factor κB (NF-κB) plays central roles in inflammatory responses and is composed of homo- or hetero-dimer of Rel family proteins, RelA (p65), c-Rel, RelB, p50 and p52 [1]. Inhibitor of κB (IκB) family members, such as IκBα, IκBβ and IκBε, sequester NF-κB dimer in the cytoplasm as an inactive complex. Upon stimulation, IκB proteins are rapidly degraded by proteasome, allowing NF-κB dimer to translocate into the nucleus, where it activates the transcription of target genes [2]. In contrast to those "classical" IκB proteins, Bcl-3, IκBζ, IκBNS and IκBη have been identified as nuclear resident IκB proteins that either positively or negatively regulate NF-κB activity [3,4]. These proteins directly modulate the transcription of a set of NF-κB-controlled cytokine genes through the binding to p50 and/or p52 subunit of NF-κB.

We previously reported that SNPs within the *NFKBIL1* gene locus, which encodes IκBL protein, are strongly associated with rheumatoid arthritis [5]. Furthermore, several autoimmune-disorders such as systemic lupus erythematosus, Sjogren's syndrome and Takayasu's arteritis were also reported to be associated with the polymorphism of the gene locus [6–8]. Since IκBL contains the region that has significant homology to the ankyrin repeat of the IκB family, it has been assumed that IκBL modulates NF-κB activity. However, the precise role of IκBL still remains elusive [9,10]. Recently, we demonstrated that the forced expression of human IκBL in mice suppressed the development of experimentally induced arthritis [11]. In the human IκBL-Tg mice, dendritic cell functions, including support for T cell activation and production of inflammatory cytokines, were impaired.

In this study, we show that IκBL inhibits NF-κB activity and inflammatory cytokine expression and that this activity requires its nuclear localization signal (NLS) sequence and ankyrin repeat domain (ANK), suggesting that IκBL is a novel nuclear IκB family member that is involved in the control of inflammatory responses.

2. Materials and methods

2.1. Reagents and cell culture

Lipopolysaccharide (LPS; sc-3535) and antibodies specific for IκBα (C-21), RelA (A), IκBL (H-225), Sp1 (PEP2) and β-actin (C-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p50 (ab7971) and α/β-tubulin (2148) were obtained from Abcam (Cambridge, UK) and Cell Signaling Technology (Danvers, MA), respectively. RAW264.7 cells (kindly provided by Dr. N.

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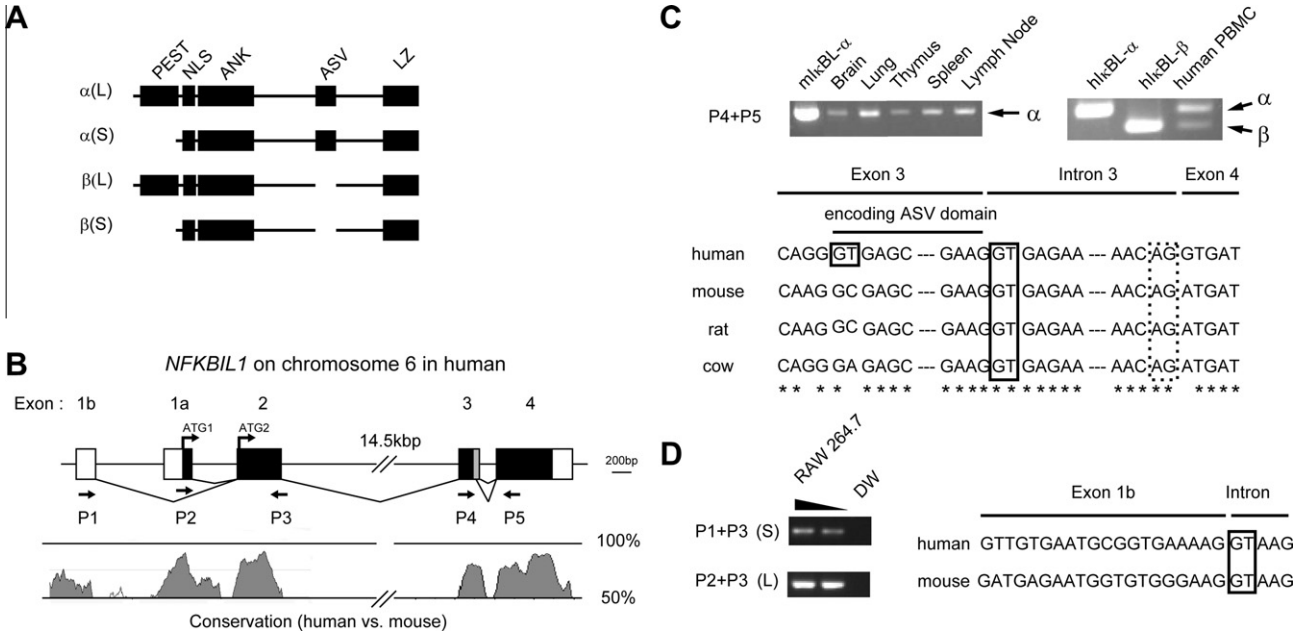

Fig. 1. Two IκBL isoforms exist in mouse cells. (A) Schematic representation of IκBL isoforms. Abbreviations: PEST, Proline, Glutamic acid, Serine and Threonine rich sequence; NLS, Nuclear Localization Signal; ANK, Ankyrin repeat domain; AVS, Alternative Slicing Variant domain; LZ, Leucine Zipper. (B) Human and mouse IκBL loci were aligned by Mulan program (http://mulan.dcode.org/). Gray histograms show conserved regions more than 70% identical between human and mouse. White, black and gray boxes represent UTR, ORF and ASV regions, respectively. P1–P5 depict the sites of designed primers. (C) RT-PCR analysis was performed to detect β isoform of IκBL using cDNAs from indicated tissues or cells (top). P4 and P5 primers were used to amplify IκBL gene fragments. IκBL-encoding plasmids were used as positive controls. Genomic DNA sequences around exon3 and 4 of IκBL gene among four species are shown (bottom). Solid and dotted rectangles mean splice donor and splice acceptor site, respectively. Asterisks represent conserved nucleotides beyond species. (D) Transcripts of shorter and longer isoforms were amplified by PCR (left). P1/P3 and P2/P3 primers were used for amplification of (S) and (L) isoforms, respectively. Genomic DNA sequence boundary of exon1b and intron are shown, and solid rectangle indicates splice donor site (right).

Kojima, Tokai Univ.), Plat-E cells and HEK293T cells were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Vectors and cell lines

Construction of vectors and establishment of stable cell lines were essentially described in Supplementary data.

2.3. Polymerase chain reaction (PCR)

Quantitative real-time PCR was performed as described previously [11]. Data were analyzed by ΔΔCt method. IκBL transcripts were amplified by PCR using Gold Taq (Applied Biosystems; Carlsbad, CA). Oligonucleotide sequences are provided in Supplementary data.

2.4. Reporter assay

RAW264.7 cells were transfected with pcDNA3 plasmid expressing IκBL-α(S) and its mutants along with pNF-κB-Luc and phRL-TK plasmid (both of which were kindly provided by Dr. S. Matsuda, Kansai Medical Univ.) using Lipofectamine with Plus Reagent (Invitrogen; Carlsbad, CA). The cells were cultured for 2 days, during the last 18 h of which they were stimulated with 100 ng/ml LPS. Luciferase activity was measured by dual-luciferase reporter system (Promega; Madison, WI). Transfection efficiency was normalized by *Renilla* luciferase activity.

2.5. Western blotting

Cells were lysed into buffer containing 20 mM Tris–HCl, pH7.4, 150 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 0.5 mM PMSF 1 mM DTT and Complete Protease Inhibitor Cocktail (Roche; Mannheim, Germany) and incubated on ice for 20 min. Cell debris was removed by centrifugation and supernatants were used as whole cell extract. For preparation of cytoplasmic and nuclear extracts, cells were lysed into TB buffer (10 mM HEPES, pH7.9, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol and 0.1% Triton X-100 and supplemented with Complete Protease Inhibitor Cocktail and 0.5 mM PMSF) and incubated on ice for 20 min. Nuclei were spun down and supernatants were used as cytoplasmic

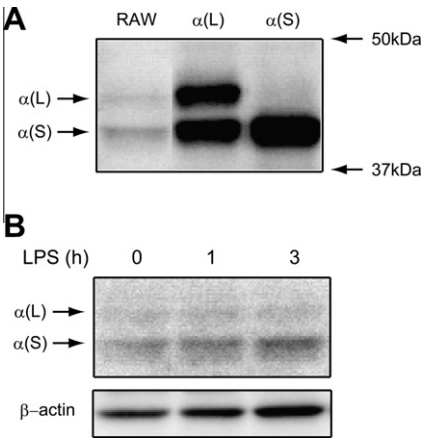

Fig. 2. IκBL-α(S) protein is predominantly expressed in RAW264.7 cells. (A) Whole cell extracts prepared from indicated cells were subjected to western blotting. The membrane was probed with anti-IκBL antibody. RAW264.7 cells carrying exon1a- and 1b-containing IκBL cDNAs were used as positive controls. Both bands corresponding to α(L) and α(S) were diminished in IκBL-knockdown RAW264.7 cells (data not shown). (B) RAW264.7 cells were stimulated with or without 100 ng/ml LPS as indicated periods and subjected to western blotting analysis.

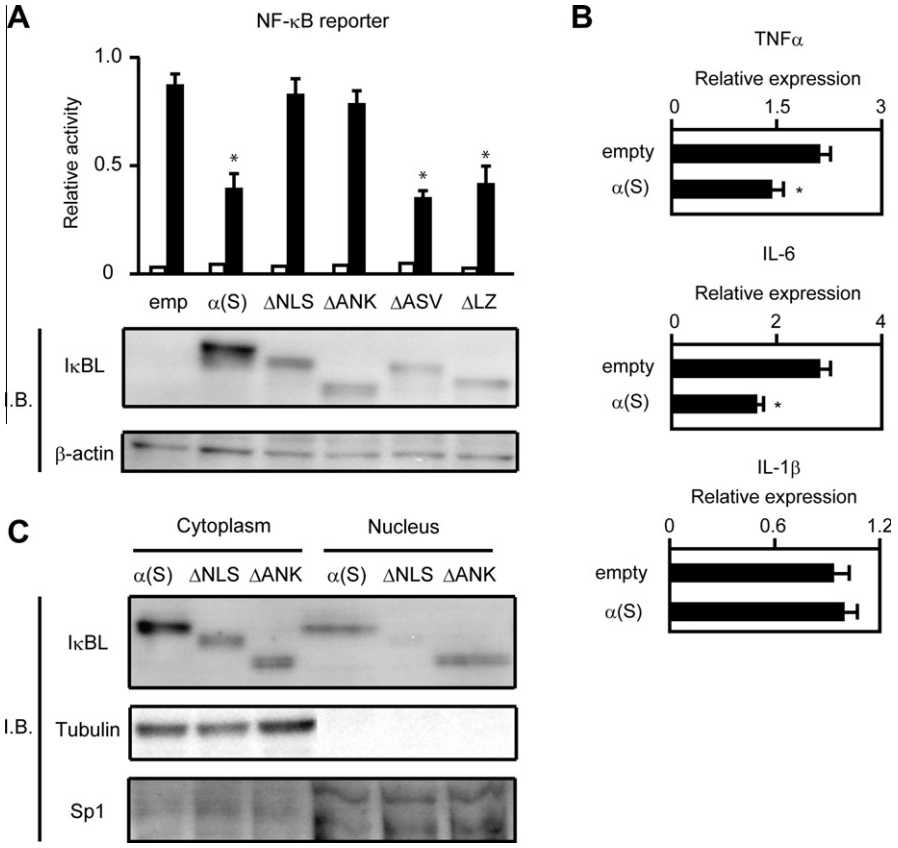

Fig. 3. IκBL suppresses NF-κB activity in an NLS- and ANK-dependent manner. (A) RAW264.7 cells were transfected with IκBL or its mutants along with NF-κB reporter and internal control reporter plasmids and stimulated with (closed bar) or without (open bar) 100 ng/ml LPS for 18 h. Luciferase activity was analyzed and normalized with *Renilla* luciferase activity. Cell lysates were subjected to western blot analysis and probed with indicated antibodies. Data are representative of at least three independent experiments and shown as averages ± S.D. $^{*}P < 0.05$. (B) RAW264.7 cells stably expressing IκBL-α(S) or empty vector control were stimulated with 100 ng/ml LPS for 3 h. TNFα, IL-6 and IL-1β expressions were analyzed by quantitative real-time PCR. Data shown are representative of three independent experiments. $^{*}P < 0.05$. (C) HEK293T cells were transfected with IκBL-α(S) and ΔNLS and ΔANK mutants. Cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis. The membrane was probed with indicated antibodies.

extracts. Nuclear proteins were extracted from nuclei by TB buffer containing 400 mM NaCl. The extracts were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were incubated with specific antibodies, followed by HRP-conjugated anti-rabbit IgG (NA9340: GE Healthcare; Little Chalfont, UK). Bound antibodies were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore; Billerica, MA).

2.6. Statistics

Data were analyzed by Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Identification of two IκBL-α isoforms in mouse cells

Previously, it was reported that human IκBL (NFKBIL1) has some different isoforms produced by alternative splicing and differential usage of the first exon [13]. The β form of IκBL is generated by alternative splicing at the 3′ end of the third exon and lacks the ASV domain, the function of which is undefined (Fig. 1A). Shorter (S) isoform utilizes exon1b as the first exon, resulting in the truncation of N-terminus PEST sequence (Fig. 1A and B). As a result, human IκBL gene encodes four different isoforms: full length, ΔPEST, ΔASV and ΔPEST-ΔASV, here termed IκBL-α(L), α(S), β(L) and β(S), respectively (Fig. 1A). We examined whether the IκBL isoforms also exist in mouse cells. Reverse-transcribed PCR (RT-PCR) analysis was performed to identify the β isoform of IκBL using specific primers (Fig. 1B, P4 and P5), which were designed to span the ASV region. IκBL-β isoform was not detected in any of the mouse tissues tested (Fig. 1C, top left), although it was detected in human PBMC (Fig. 1C, top right, lower band) as described elsewhere [9]. Comparison of genomic DNA sequence among the four species revealed that the alternative splice donor site (GT dinucleotides) was only seen in primates; the site was substituted to GC or GA in mouse, rat, cow and so on (Fig. 1C, and data not shown). These data indicated that IκBL-β isoform is absent in mouse cells.

Next, we surveyed the PEST sequence-truncated "S" isoform of IκBL in mouse cells. Since the nucleotide sequence of exons in the IκBL gene locus is well conserved beyond species (Fig. 1B, and data not shown), putative exon1b of mouse IκBL was assigned to the −670 to −544 bp upstream of its exon1a. RT-PCR analysis revealed that exon1b-containing transcript was expressed in mouse macrophage cell line RAW264.7 cells (Fig. 1D left, P1 + P3) and the splicing donor site was identified by DNA sequence analysis (Fig. 1D, right). There were no in-frame ATG methionine codons within this alternative exon1b (data not shown). Thus, it is assumed that exon1b-containing transcript is translated from the second ATG codon residing in exon2 and encoding N-terminally truncated isoform lacking the PEST sequence, while full-length protein is translated from the first ATG codon within exon1a (Fig. 1B).

To confirm the expression of IκBL isoform proteins, exon1a- and exon1b-containing cDNAs were transduced into RAW264.7 cells. Notably, exon1a-containing cDNA produced two differently-sized

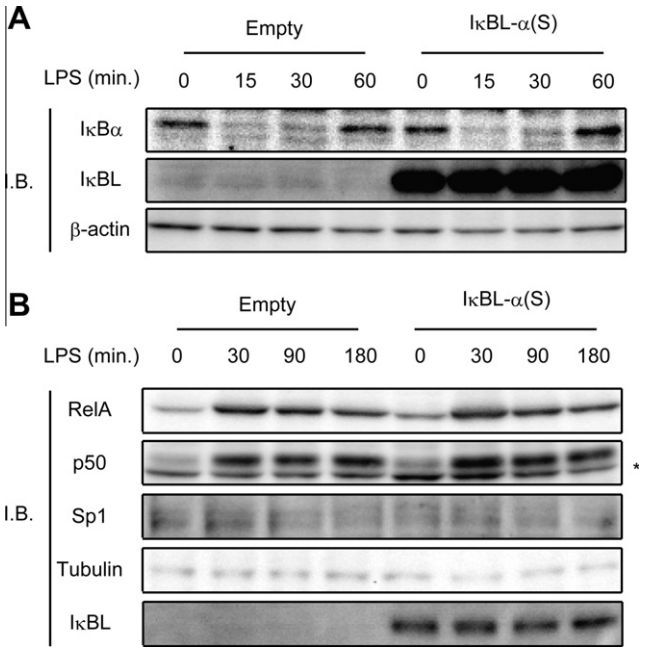

Fig. 4. NF-κB activation in the cytoplasm was not affected by IκBL. Control vector- and IκBL-α(S)-expressing RAW264.7 cells were stimulated with 100 ng/ml LPS for indicated periods. Whole cell extracts (A) and nuclear extracts (B) were resolved by SDS–PAGE and immunoblotted with indicated antibodies. Asterisk indicates non-specific band.

proteins, while exon1b-containing cDNA produced only the shorter form (Fig. 2A). It is suggested that both the 1st and 2nd ATGs are used as the start codon when exon1a-containing transcript is translated (Fig. 1B), resulting in the synthesis of α(S) isoform, as well as α(L) isoform. Endogenous IκBL proteins were also detected in RAW264.7 cells and slightly increased after exposure to LPS (Fig. 2A and B). IκBL-α(S) isoform was predominantly expressed in both LPS-stimulated and unstimulated RAW264.7 cell (Fig. 2), although transcripts of IκBL-α(L) were over three times greater than those of α(S) (data not shown). Since the PEST sequence is known to play a role as a target for proteolysis (Ref. [12]), it is assumed that the PEST sequence-truncated "S" isoform is more stable and predominantly exists in the cells.

3.2. IκBL inhibits NF-κB activity and inflammatory cytokine expression

IκBL contains ANK, which exhibits high homology to ANK of IκB proteins, raising the possibility that IκBL modulates NF-κB activity. Upon LPS stimulation of RAW264.7 cells, IκBL-α(S)-transduced cells significantly suppressed NF-κB-driven luciferase activity compared with empty vector control-transduced cells (Fig. 3A). This result suggested that IκBL functions as an inhibitor of NF-κB. It is well known that the expression of inflammatory cytokines is largely dependent on NF-κB activity [2]. Consistent with the result of the luciferase assay (Fig. 3A), TNFα and IL-6 transcriptions, but not IL-1β transcription, were suppressed by the forced expression of IκBL-α(S) (Fig. 3B). It is suggested that IκBL inhibits part, if not all, of NF-κB-driven gene transcription.

3.3. Nuclear localization signal (NLS) sequence and ankyrin repeat domain (ANK) are required for IκBL activity

In addition to ANK, IκBL-α(S) contains NLS, ASV and LZ regions (Fig. 1A). Then, we examined the contribution of these regions to the IκBL activity. Truncation of NLS (ΔNLS) and ANK (ΔANK) within IκBL-α(S) failed to inhibit NF-κB-driven and IL-6 promoter-driven luciferase activity (Fig. 3A, and data not shown), while comparable activity was observed in ASV(ΔASV; relative to β(S) isoform in human)- or LZ (ΔLZ)-lacking mutants (Fig. 3A). IκBL is localized both in the cytoplasm and in the nucleus [13]. The NLS region is required for nuclear localization, since ΔNLS is detected only in the cytoplasm, but not in the nucleus (Fig. 3C). In contrast, truncation of ANK domain within IκBL did not alter its subcellular localization (Fig. 3C). Thus, ANK domain is required for the activity irrespective of its localization. Collectively, it seems clear that IκBL is a novel member of the nuclear IκB family.

3.4. Nuclear translocation of NF-κB dimer occurs normally even in the forced expression of IκBL

In resting cells, NF-κB dimer forms an inactive complex with conventional IκBs such as IκBα. Following activation via TLR, cytoplasmic IκB proteins are rapidly degraded, allowing NF-κB dimer to translocate into the nucleus [2]. We examined whether NF-κB activation cascade in the cytoplasm is influenced by IκBL. In both control and IκBL-expressing RAW cells, IκBα was completely degraded within 30 min and re-synthesized at 60 min after LPS stimulation (Fig. 4A). Consistent with the kinetics of IκBα degradation, comparable amounts of NF-κB dimer (composed of RelA and p50) were observed in the nucleus in empty control and IκBL-expressing RAW cells (Fig. 4B). Cytoplasmic RelA and p50 levels did not change throughout the periods tested (data not shown). Therefore, IκBL does not affect the nuclear translocation of NF-κB. Taken together, these results highlight the fact that IκBL is a novel member of nuclear IκBs, and that it negatively regulates transcription of some genes controlled by NF-κB in the nucleus.

4. Discussion

In this study, we showed that IκBL, predominantly α(S) isoform, inhibited NF-κB activity. Similar to nuclear IκB protein family IκBζ and Bcl-3 (Refs. [14] and [15]), IκBL reduced NF-κB activity in an NLS and ANK regions-dependent manner without affecting the nuclear translocation of RelA and p50. These findings support the notion that IκBL is a novel member of the nuclear IκB protein family.

It was reported that IκBNS and Bcl-3 inhibit the transcription of IL-6 and TNFα, respectively [15,16]. Unlike "classical" IκB proteins, IκBNS and Bcl-3 exert their roles in the nucleus to stabilize DNA-bound p50 homo-dimer, which is known to be a transcriptional inactive complex, resulting in the prevention of RelA-containing active dimer recruitment to the promoter of inflammatory cytokine genes [15,16]. In our preliminary experiment using chromatin immuno-precipitation technique, the forced expression of IκBL reduced the recruitment of RelA on the IL-6 and TNFα promoters (unpublished data), assuming that IκBL regulates cytokine gene expression by controlling the binding of active NF-κB complex to DNA.

It is suggested that IκBNS and Bcl-3 bind to p50 through the ANK region [17]. Although ANK region of IκBL is required for its activity, we failed to detect the binding of IκBL to p50 and p52 (data not shown). Since the amino acid sequence of IκBL ANK region slightly differed from that of Bcl-3 and IκBNS (Ref. [18]), it is plausible that IκBL binds to some other NF-κB subunit. It seems intriguing to hypothesize that IκBL binds to RelB and support its inhibitory function, since RelB is reported to play an inhibitory role in cytokine gene transcription [19,20]. Further investigations are needed to clarify the underlying mechanisms for IκBL-mediated

inhibition of NF-κB-dependent gene expression. Transcriptional regulation of inflammatory mediators by IκBL may provide new insights into how inflammatory disorders develop.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.10.024.

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